

Cloning and *in vitro* Anti-mycobacterial Activity of Lectin Protein In Combination with Streptomycin to Increase Sensitivity Against *Mycobacterium tuberculosis*

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ABSTRACT

Research on anti-mycobacterial bioactivity of lectin protein from red algae Gelidium amansii of Barang Lompo Island was conducted. Pre-purification of protein using fractional method, showed bioactivity against Mycobacterium tuberculosis in whole extract and with the saturation level of ammonium sulfate of 40-60%. Further purification of this protein fraction using column chromatography system followed by protein sequencing, indicated the pure protein as lectin, that migrates as a single-band on SDS-PAGE with molecular weight of 21 kDa. Based on amino acids partial sequence, we cloned and sequenced cDNA encoding lectin protein. It consists of 552 nucleotides encoding 183 amino acid residues including a putative initiation Met. To obtain it in large amounts, the coding sequence of lectin was cloned into pGEX-2TK vector and expressed as a lectin fusion protein in Escherichia coli. Recombinant lectin protein alone did not show any growth inhibitory effect on M. tuberculosis, but could increase the sensitivity of this pathogen to drug streptomycin. These research suggests that anti-mycobacterial lectin protein in combination with streptomycin may play an important role in host defence against M. tuberculosis strain H37Rv.

Keywords: Red algae, *Gelidium amansii*, Lectin, Recombinant protein, Anti-mycobacterial activity

INTRODUCTION

Indonesia, a maritime country with ocean area of 75% covering the country, has abundant source of marine biota; among them are a variety of marine algae species. Some species have been reported to contain bioactive compounds with wide applications in the pharmaceutical industries (Sheih *et al.* 2010). In parallel to the trend of disease pattern changes such as the resistance of disease germs towards a certain medicine, efforts to find new medicines are necessarily to be carried out. Until recently, marine natural resources have not been optimally utilized. Therefore, any efforts to identify potential bioactive compounds from marine natural resources will be of great interest (Blunt *et al.* 2007).

On the other hand, worldwide, tuberculosis (TB) remains the most frequent and important infectious disease causing morbidity and death. One-third of the world's population is infected with *Mycobacterium tuberculosis* (MTB), the etiologic agent of TB. The World Health Organization (WHO) estimates that about eight to ten million new TB cases occur annually worldwide and the incidence of TB is currently increasing (WHO. 2005). In this context, TB is in the top three, with malaria and HIV being the leading causes of death from a single infectious agent, and approximately two million deaths are attributable to TB annually. In particular, pulmonary TB, the most common form of TB, is a highly contagious and life-threatening infection. Moreover, enhanced susceptibility to TB in HIV-infected populations is another serious health problem throughout the world. In addition, multidrug-resistant TB (MDR-TB) has been increasing in incidence in many areas, not only in developing countries but also industrialized countries as well, during the past decade, including in Indonesia (WHO. 2005). Very recently, patterns of resistance to commonly used anti-tuberculosis drugs among *M. tuberculosis* complex isolates from patients attending government urban TB diagnostic in Makassar, the capital of the South Sulawesi Province in Indonesia, was found the highly potential risk factors for MDR-TB (Massi *et al.* 2011). In order to tackle these new situations, it is necessary and important to develop not only new treatment guidelines, but also new anti-mycobacterial drugs for efficacious clinical control of TB patients.

Protein as anti-mycobacterial medicine has been promising since it can be well accepted by human body and has few side effects. Therefore, research on the use of protein and or peptides as a source of medicine is tremendously growing. Moreover, the genes in a group of protein can be cloned to produce medicine in large scale using genetic engineering approach. Antimicrobial protein/peptides (APs) from eukaryotes are components of the innate immune defense and display potent antimicrobial activity against a variety of microorganisms (Liao *et al.* 2003). Lectins are proteinaceous substances that are widely distributed in animals, plants, and microorganisms have been found to contain hemagglutinating activity (Etzler 1986). Since the report of Boyd *et al.* (1966), numerous studies have indicated that marine algae contain lectins capable of binding specific carbohydrates to produce unique biological activities, such as the aggregation of erythrocytes, yeasts, bacteria, and various unicellular algae (Hori *et. al.* 1986; Hori *et. al.* 2007). In marine algae, lectins play an important role in recognition and adherence of gametes during sexual reproduction (Kim and Kim 1999). Marine organism lectins including from algae seem to be especially interesting for biological applications, because they are in small molecules, induce minor immunogenicity in host cells, possess great stability on account of their several disulfide bridges, and show high specificity for complex glycoconjugates, such as the

ones present on cell surfaces (Sampaio *et al.* 1998). Other biological and ecological activities of algae lectins are probably involved in symbiosis and defense as exhibited by lectins of terrestrial plants (Etzler 1986), and other marine invertebrates (Kilpatrick 2002; Olafsen 1996). However, these results have not been clearly proven. APs appear to interact and cross cell envelope membranes of microbes and kill cells by a complex mechanism that involves action on more than one anionic target. Antimicrobial protein/peptides lectin, protegrin-1, human alpha-defensin-1, and PR-39 are known to be active against vibrios and mycobacterium (Liao *et al.* 2003; Fattorini *et al.* 2004; Linde *et al.* 2001), the organism responsible for tuberculosis, but no studies on the combination between APs and clinical anti-MTB drugs have been performed.

Thus, purification and characterization of lectins from a variety of plant and animal species interests researchers in the field of biochemistry and molecular biology. The more that is known about the lectins, the wider are the applications of this type of proteins. In this study, we report for the first time the purification, characterization, and cDNA cloning of a new anti-mycobacterial lectin protein isolated from red algae of *Gelidium amansii*. Next we tested the activity of lectin recombinant protein alone and in combination with the anti-MTB drug streptomycin (SM), against *Mycobacterium tuberculosis* (MTB) strain H37Rv (ATCC 27294) to increase sensitivity of anti-mycobacterial drugs.

MATERIALS AND METHODS

Materials

Red algae *Gelidium amansii* was collected in the region of Barang Lompo Island, South Sulawesi Province, Indonesian territory. pGEX-2TK vector, CM-cellulose, Sephadex G-100, *E. coli* BL21 competent cells, glutathione agarose beads, and Trizol reagent kit were purchased from Amersham Pharmacia Biotech. Mono S resin was purchased from GE Healthcare (Hong Kong). Streptomycin, INH, ammonium sulphate, and lysozyme were purchased from Sigma.

Procedures

Isolation and purification of anti-mycobacterial lectin protein from red algae

Five hundred grams of fresh red algae *Gelidium amansii* were homogenized with waring blender using 500 mL of buffer solution A (Tris-HCl 0,02 M pH 7,3, NaCl 0.2 M, CaCl₂ 0,01 M, β -mercaptoetanol 1 %, Triton X- 100 0,5 %). Next, resultant was filtered with Buchner. The

filtrate obtained was frozen and reliquified, 2 or 3 times, centrifuged at 10,000 X g at 4°C for 30 minutes, and the obtained supernatant was stored in a refrigerator before being tested for anti-mycobacterial agent and further purification steps. The supernatant (whole extract) containing protein and having anti-mycobacterial activities was then fractionated using ammonium sulphate at saturated levels of 0 – 20%, 20 – 40%, 40 – 60%, and 60 – 80%, respectively.

The precipitates obtained after fractionation at various saturation levels of ammonium sulphate was then suspended in 25 mL of buffer B (Tris-HCl 0.02 M pH 7.3, NaCl 0.2 M, CaCl₂ 0.01 M), and dialysed in buffer solution C (Tris-HCl 0.01 M pH 7.3, NaCl 0.2 M, CaCl₂ 0.01 M) using selophan pocket (Sigma) until colorless buffer was obtained. After dialysis, each protein fraction was subjected to anti-mycobacterial activity.

The protein fraction of ammonium sulphate with containing anti-mycobacterial activity (100 mg) was dissolved in 5 ml of 20 mM Tris-HCl buffer (pH 7.3) and centrifuged (12,000 g, 5 min). The supernatant were loaded on a CM-cellulose gel column 5 × 18 cm (Amersham Pharmacia Biotech, Sweden) which had been equilibrated with the same buffer. After eluting unbound proteins, bound proteins with hemagglutinating activity were eluted with 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.3), dialyzed, lyophilized, and then subjected to gel filtration using a Sephadex G-100 (Pharmacia, USA) column (1.5 × 134 cm). After eluting the unbound proteins, bound proteins with hemagglutinating activity were eluted with the same buffer, dialyzed, lyophilized, and then subjected to ion exchange chromatography by Fast Protein Liquid Chromatography (FPLC) using an AKTA purifier (GE Healthcare, Hong Kong, China) on Mono S column (GE Healthcare, Hong Kong) in 20 mM Tris-HCl buffer (pH 7.3). The eluted proteins were monitored at 280 nm by UV Spectrophotometer and hemagglutinating activity test was determined with trypsin-treated sheep erythrocytes according to the method of Hori *et al.*, (1986). After each step, the protein profiles of the active fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 14% separating gel. Proteins were detected by Coomassie Brilliant Blue R staining.

Protein quantitative concentration

The calculation of protein concentration at different purification steps was determined based on Lowry method (Lowry *et al.* 1955) using Bovine Serum Albumin (BSA) as a standard.

Amino acid sequencing and gene cloning encoding lectin protein

Native lectin protein (0.5 mg) was reduced by dithiothreitol, S-carbox-amidomethylated and digested with endoproteinase C (substrate:enzyme = 50:1, w/w) in 40 µl of 10

mM Tris- HCl (pH 7.5) at 37°C for 2 h. Each digest was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a TSK gel ODS 120T column (4.6 X 250 mm²; Tosoh , Tokyo, Japan) using a linear gradient of acetonitrile (0–80 % in 70 min) in 0.1 % trifluoroacetic acid (TFA). The N-terminal amino acid sequences of lectin and its proteolytic peptide fragments were determined using a protein sequencer (Applied Biosystems Division, Model 473A, Perkin-Elmer).

Cloning and sequencing of cDNA encoding lectin from Red algae Gelidium amansii

Based on conserved amino acid sequences (PLQGRSQKTE and GNEDCLDLRT) from partial amino acid sequence of native lectin protein from red algae *Gelidium amansii* deduced from their cDNAs, sense and antisense degenerate oligonucleotide primer pairs L1 and L2 containing sequences 5'-cctcttcagggtaggagccagaaaaccgag-3' and 5'-cgtcctgagggtcaaggcagtcctcgttcc-3', respectively, were constructed. A PCR product of 422 nucleotides, corresponding to a part of the coding regions of red algae *Gelidium amansii* lectin was prepared from the *Gelidium amansii* cDNAs using the two degenerate primers. To obtain full-length Ga Lectin cDNAs, using the resultant PCR product as a probe, we screened the *Gelidium amansii* λZAP II cDNA library, using poly(A) mRNAs prepared by Trizol reagent kit from the red algae *Gelidium amansii* cells, according to Ahmad *et al.*, (1999). The entire nucleotide sequences of both strands of the largest cDNA insert were sequenced by the dye terminator method (Applied Biosystems Division, Model 310, Perkin-Elmer).

Expression and production of the recombinant GST-lectin fusion protein in E.coli

The GST Gene Fusion System (Pharmacia Biotech Inc.) was used to express lectin protein following the procedure of Ahmad *et al.*, (1999). An overnight culture of *E. coli* BL21 strain containing pGEX-2T lectin cDNA was diluted 1:10, grown at 37°C to an optical density (OD)₆₀₀ of 1.0, and induced with 60 μM IPTG (isopropyl-1-thio-,3-D-galactoside) for 3 h at 37°C. The culture was centrifuged and the pellet suspended in 10 ml of lysis buffer (Tris-HCl 0.02 M pH 7.3, NaCl 2 M, CaCl₂ 0.01 M, β -mercaptoetanol 1 %, Triton X- 100 0.5 %), containing 0.1% (v/v) phenylbenzosulfonyl fluoride and 1 mg/ml lysozyme. Following a 15-min incubation on ice, dithiothreitol and Sarkosyl were added to 5 mM and 1.5% final volumes, respectively. The sample was sonicated for 2 min on ice in a water bath sonicator, centrifuged, and Triton-X 100 (2% final volume) was added to the supernatant. Next, glutathione agarose beads were added to the supernatant and incubated for 20 min at 4°C with gentle rotation. The beads were collected by centrifugation at 3,000 g for 2 min and washed five times with cold

1X PBS buffer. The fusion protein (GST-Lectin) was eluted with 4 mL of 20 mM glutathione in 20 mM Tris-HCl, pH 7.3, and the resultant eluate was concentrated with a Millipore membrane, followed by the addition of glycerol to a final concentration of 20%. The samples obtained were resolved by 14% SDS-PAGE, according to Sambrook *et al.*, (1989). The recombinant GST-lectin fusion protein concentrations were determined by the Lowry method (Lowry *et al.* 1955) using BSA as a standard. The anti-mycobacterial activity of the recombinant GST-lectin fusion protein was determined as described below.

Anti-mycobacterial activity assays

For the initial screening, standard colony assays on Löwenstein-Jensen (LJ) medium were performed to assess the anti-mycobacterial activity of whole extract, ammonium sulphate fraction, native and recombinant lectin protein with the final concentrations of 10 µg/mL, respectively, using the strain *M. tuberculosis* H37Rv (ATCC 27294) and solvent 0.02 M Tris-HCl pH 7.3 as negative control and combination of streptomycin plus INH as positive control (Yu *et al.* 2011). For testing the anti-mycobacterial activity of recombinant lectin protein and in combination with streptomycin, the strain *M. tuberculosis* H37Rv (ATCC 27294) were grown on Middlebrook 7H10-OADC (oleic acid, albumin, dextrose, catalase) (Difco Laboratories) at 37 °C for 3 weeks until midlog phase. Tenfold serial dilution (20 µL in 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) were streaked on Middlebrook 7H10-OADC agar plate containing solvent (0.02 M Tris-HCl pH 7.3) as negative control, streptomycin (5 and 10 µg/mL), and recombinant lectin and or native lectin (5 and 10 µg/mL) were indicated. Plates were incubated at 37 °C and inhibition growth were documented after 2 to 3 weeks. The assays was conducted in duplicates and repeated three times to produce representative experimental data.

RESULTS AND DISCUSSION

Results

Isolation, pre-purification, and determination of lectin protein concentration

The whole extract having anti-mycobacterial activity was fractionated using ammonium sulphate at the saturation level of 0 – 20%, 20 – 40%, 40 – 60%, and 60 – 80%. The addition of ammonium sulphate salts from low to high concentration resulted in different protein types precipitated. Higher concentration of ammonium sulphate caused a lot of hydrophobic functional groups neutralized by ammonium sulphate salts so that water could no longer bond

resulting in the decrement of protein solubility in water causing the protein to be precipitated. Data on distribution pattern of whole extract and protein fraction at fractionation of various saturation levels of ammonium sulphate is shown in Table 1. Protein concentration from whole extract of red algae *Gelidium amansii* was 1.2 mg/mL with the total amount of protein 492.0 mg from the whole extract volume 410 mL. The highest protein concentration of 5.6 mg/mL was found at fraction of ammonium sulphate 40-60%, whereas the lowest protein concentration (1.5 mg/mL) was found at ammonium sulphate fraction 60-80%.

Table 1. Distribution of protein concentration in whole extract and protein fraction at fractionation of various saturation levels of ammonium sulphate from red algae *Gelidium amansii*

No.	Protein Fractions	Fraction Volume (mL)	Protein Concentration (mg/mL)	Total Protein (mg)
1	Whole extract	410	1.2	492.0
2	0-20 %	25.0	4.5	112.5
3	20-40 %	25.0	1.6	40.0
4	40-60 %	25.0	5.6	140.0
5	60-80 %	25.0	1.5	37.5

Screening of native anti-mycobacterial lectin protein

At the first experiment of anti-mycobacterial test, it was obvious that each protein fraction from red algae *Gelidium amansii* at various levels of saturated ammonium sulphate exhibited anti-mycobacterial activity as indicated by 40-60% fraction protein, and whole extract samples with no colony growth of *M. tuberculosis* on Löwenstein-Jensen medium. However, other fractions did not display anti-mycobacterial activity (Figure 1). This finding was in accordance with previous study that antibacterial and antitumor protein from marine algae was found from 40-60% fraction level of saturated ammonium sulphate (Liao *et al.* 2003; Yan, *et al.* 2009). Hence, these fractions were very promising and were selected for further efforts on purification and cloning of the gene encoding lectin protein compound for future experiment.

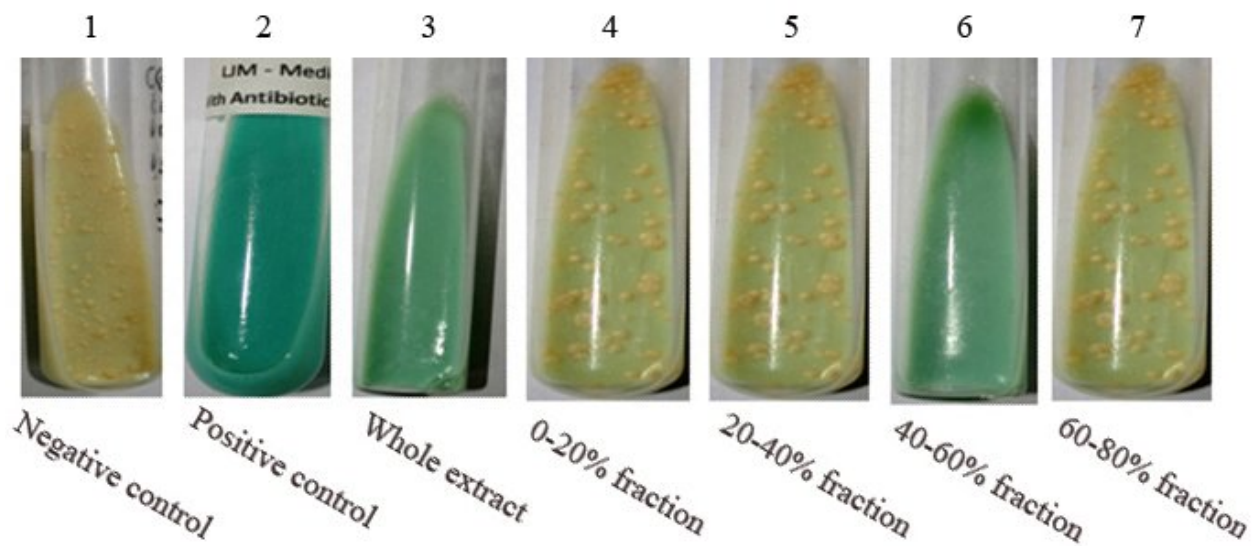


Figure 1. The colony growth of *M. tuberculosis* on Löwenstein-Jensen medium containing protein from red algae *Gelidium amansii* at various level of percentanges saturated of ammonium sulphate as indicated.

Purification of native anti-mycobacterial lectin protein from red algae Gelidium amansii

Protein fraction (100 mg) from ammonium sulphate fractionation at 40-60% fraction was applied on a CM-cellulose cation-exchange column (5×18 cm). Proteins bound to the CM were eluted using a gradient from 0.1 to 0.5 M Tris-HCl pH 7.3 at a flow rate of 1 mL/min, and 60 fractions were collected. The fractions eluting between tube number 24 to 28 demonstrated hemagglutinating activity (Figure 2A). Next, the protein fraction from CM-sellulose cation-exchange column that demonstrated hemagglutinating activity were applied to gel filtration chromatography on Sephadex-G100, and the resulting three peaks were collected. The first and third peaks showed no hemagglutinating activity, while the second peak indicated having hemagglutinating activity (Figure 2B). The second peak was further purified by FPLC on Mono S column using a shallower acetonitrile gradient, giving the single peak of pure protein (Figure 2C). Based on the SDS-PAGE (14%), the molecular weight of the purified lectin protein was estimated to be approximately 21 kDa (Figure 2D), and this result was consistent with that calculated by Bioedit 7.9 software, giving the molecular weight of 20,822 Da (result not shown).

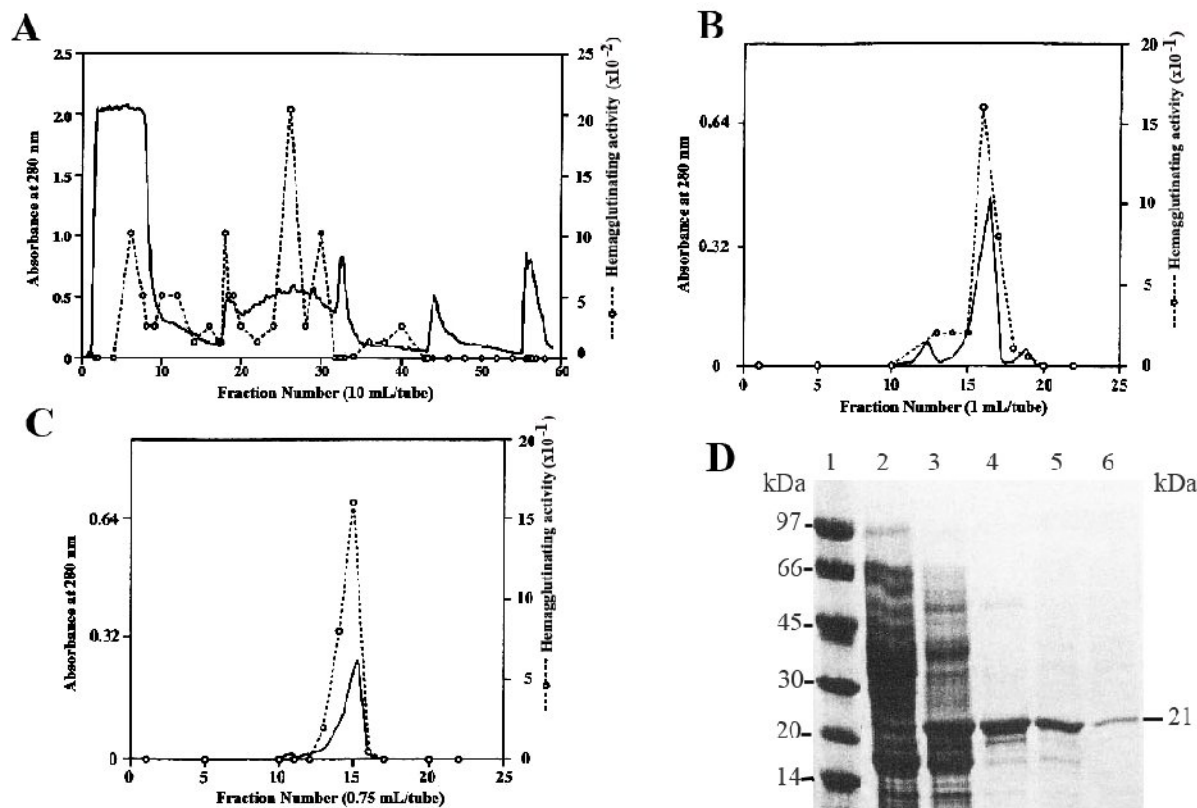


Figure 2. Purification of a novel anti-mycobacterial lectin protein from the red algae *Gelidium amansii* (A) Cation-exchange chromatography of protein 40-60% fraction from ammonium sulphate fractionation on a CM-cellulose cation-exchange column (5×18 cm). (B) Gel filtration chromatography of pooled active fractions from the cation-exchange column on a Sephadex G-100 column (1.5 X 134 cm). (C) Pooled active fractions of peak two from gel filtration chromatography in (B) was purified by FPLC on mono S column using a shallower acetonitrile gradient. The elution profile was monitored at 280 nm. (D) SDS-PAGE (14%) analysis of the active fractions obtained at each purification step. The gel was stained with Coomassie Brilliant Blue R. Lane 1, Protein marker in kDa; lane 2, whole extract materials; lane 3, protein 40-60% fraction from ammonium sulphate fractionation; lane 4, pooled active fractions from CM-Cellulose chromatography; lane 5, pooled active fractions (peak 2) from gel filtration chromatography; lane 6, single peak from the FPLC system.

Amino acid sequencing and gene cloning encoding lectin protein

Enzymatic digests of native lectin protein with endoproteinase C were separated by RP-HPLC and subjected to protein sequencing, resulting in the partial N-terminal and an internal fragment of amino acid sequence of lectin, identified to be sequence PLQGRSQKTE and GNEDCLDLRT, respectively. To complete the amino acid sequence of lectin, cDNA sequencing was carried out by RT-PCR and plaque hybridization. Based on the partial amino acid sequence above, the algae lectin specific probe of 422 bps (Figure 3 in lane 4) was identified with oligonucleotides corresponding to peptides derived from pure native lectin, using PCR and DNA sequence analysis. The probe was used to screen a whole extract cDNA library of red algae *Gelidium amansii* (about 200,000 recombinant phages), and one positive clone with the a largest

cDNA insert was chosen and subjected to restriction mapping followed by sequence determination. The nucleotide and deduced amino acid sequences are shown in Figure 4. The cDNA included 599 nucleotides with an encoding region of lectin protein of 552 nucleotides. The open reading frame for the cDNA encoded for a native protein of 183 amino acid residues with a molecular weight of 20,822 Da (as calculated by Bioedit software) (result not shown). The candidate for an initiation codon atg was found at nucleotide position 21. The stop codon

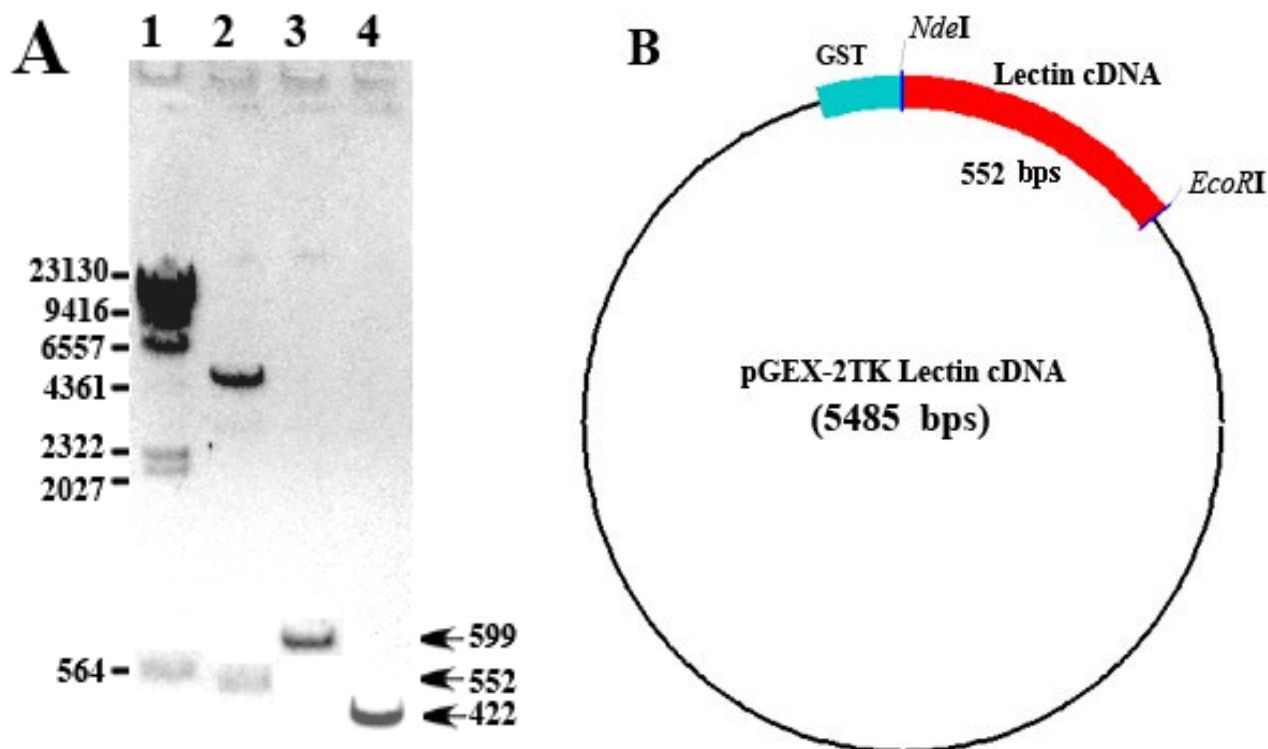


Figure 3. Schematic representation of the lectin gene synthesis by the RT-PCR and plaque hybridization method and the construction of pGEX-2TK fusion vector. A) Agarose gel electrophoresis of DNA fragment of RT-PCR product and recombinant plasmid. Lane 1, DNA lambda/*Hind*III marker; lane 2, two fragments DNA containing pGEX-2TK vector (4963 bps) and insert of Lectin DNA minus 5' untranslation region (552 bps) digested by *Nde*I/*Eco*RI restriction enzymes of recombinant plasmid in B; lane 3, largest cDNA insert (599 bps) from positive clone; and lane 4, RT-PCR product of lectin DNA as probe (422 bps). B) Restriction map of pGEX-2TK lectin cDNA plasmid.

at position 550 was followed by polyadenylation signal agaaaa, starting at position 569. Amino acid sequences of the isolated peptides derived from algae lectin corresponded exactly to the protein sequence deduced from the cDNA sequence, clearly indicating that the isolated cDNA clone codes for lectin protein.

																caccaccgaaccagcagcat	-20
1	atg	cta	atc	cat	ttg	agc	aat	aca	act	cag	gta	tgc	atc	tca	tct		45
1	M	L	I	H	L	S	N	T	T	Q	V	C	I	S	S		15
46	ttg	gtg	atc	<u>cct</u>	<u>ctt</u>	<u>cag</u>	<u>ggg</u>	<u>agg</u>	<u>agc</u>	<u>cag</u>	<u>aaa</u>	<u>acc</u>	<u>gag</u>	cac	gta		90
16	L	V	I	<u>P</u>	<u>L</u>	<u>Q</u>	<u>G</u>	<u>R</u>	<u>S</u>	<u>Q</u>	<u>K</u>	<u>T</u>	<u>E</u>	H	V		30
91	ctg	ttg	aag	cgg	agc	tac	ctg	tgg	ggg	acg	ttg	ccc	agt	gac	aac		135
31	L	L	K	R	S	Y	L	W	G	T	L	P	S	D	N		45
136	atc	ctc	gcc	tgt	gac	agg	ccc	tca	gat	tat	cta	tgt	ccc	tac	gta		180
46	I	L	A	C	D	R	P	S	D	Y	L	C	P	Y	V		60
181	tat	aag	caa	acg	cat	gac	gag	tgc	ttc	tat	ctg	gac	gag	gtc	aag		225
61	Y	K	Q	T	H	D	E	C	F	Y	L	D	E	V	K		75
226	tca	aga	atg	aac	caa	gac	cga	cag	tac	tgc	cag	ctg	ctg	cag	ggc		270
76	S	R	M	N	Q	D	R	Q	Y	C	Q	L	L	Q	G		90
271	gac	ctg	gcc	aca	ccc	agg	aac	gtc	caa	gga	cca	cag	tcc	ttc	gtc		315
91	D	L	A	T	P	R	N	V	Q	G	P	Q	S	F	V		105
316	atc	gac	act	aat	ggc	tac	gtg	acc	gag	cag	tgg	ctg	gga	gca	acc		360
106	I	D	T	N	G	Y	V	T	E	Q	W	L	G	A	T		120
361	gac	cag	tcc	tcg	gag	gga	acg	tgg	aac	tgg	ctg	tgg	atc	cac	ccc		405
121	D	Q	S	S	E	G	T	W	N	W	L	W	I	H	P		135
406	tca	cat	tcg	gac	tgg	gcc	ggc	ggg	cag	cct	gat	aaa	gcc	gga	<u>gga</u>		450
136	S	H	S	D	W	A	G	G	Q	P	D	K	A	G	<u>G</u>		150
451	<u>aac</u>	<u>gag</u>	<u>gac</u>	<u>tgc</u>	<u>ctt</u>	<u>gac</u>	<u>ctc</u>	<u>agg</u>	<u>acg</u>	aag	ata	agg	gtt	gac	ggc		495
151	<u>N</u>	<u>E</u>	<u>D</u>	<u>C</u>	<u>L</u>	<u>D</u>	<u>L</u>	<u>R</u>	<u>T</u>	K	I	R	V	D	G		165
496	aac	gat	tac	caa	tgc	gga	gtg	gct	agt	ata	ttc	gtg	tgc	cag	tac		540
166	N	D	Y	Q	C	G	V	A	S	I	F	V	C	Q	Y		180
541	aat	gct	cgc	tag	gagggc	cacgaa	agtcag	agagaaaa	aaaaa								579
181	N	A	R	End													183

Figure 4. The cDNA and deduced amino acid sequence of lectin protein. Fragment DNA (422 bps) as probe synthesis by RT-PCR amplification of total red algae *Gelidium amansii* cDNAs was inserted into pGEX-2TK vector to yield pGEX-2TK Lectin recombinant plasmid. The nucleotide sequence was analyzed by the dideoxy chain-termination method. The result of N-terminal and an internal fragment of amino acid sequencing obtained from protein sequencing is indicated in box and the two primers L₁ and L₂ oligonucleotide were obtained based partial amino acids sequencing is underlined.

Based on Bioedit 7.9 software of the amino acids sequence, the sequence revealed high homology with the Pm_lectin protein from crustacea (68%), but low homology with the Pp_lectin protein from mollusca (24%), and Aj_lectin protein from chordata (22%) (Figure 5). This results clearly indicated that the fragment DNA isolated from red algae *Gelidium amansii* was a gene encoding lectin protein, and could be amplified using the generated primer pairs from

the partial amino acid sequence of native lectin protein. Therefore, future intensive research is required to trace the existence of this gene and its product, especially to determine another bioactivity to strengthen the structural and functional analysis of lectin protein, since the lectin protein also possesses anticancer (Ahmad and Massi, 2011, *in preparation*) and antioxidants activity (Sheih et al. 2010).

	10	20	30	40	50
Ga_Lectin	-MLIHLSNTT	QVCISSLVIP	LQGRSQKTEH	VLLKRSYLWG	TLPSDNLILAC
Aj_Lectin	--MVSFPMSN	LIFVAVLS--	-----	-----	-----GLTLV
Pm_Lectin	-MLTHLAIFA	LVAVSISGQP	LQGRSQKTEL	LVAVGILEEQ	VQDVKQKVIA
Pp_Lectin	MLVIAVLMFC	VPSLSALRES	DVEEGK----	-----	-----TSILNC
Consensus	: .	::			
	60	70	80	90	100
Ga_Lectin	DRPSDYLCPY	VYKQTHDECF	YLDEVKSRMN	QDRQYCQLLQ	--GDLATPRN
Aj_Lectin	SEQCADTCPE	GWKGFNGCCY	KHFDLLKNWR	EAEFYCMIRG	--GHLASVHS
Pm_Lectin	LPNSGALCPY	PYKQVLDECF	YLSKVKLNNW	QARQYCQGMQ	--GDLATPRN
Pp_Lectin	PAQYKISLID	AKYGLHNRFV	TATKKAAALC	NGKKQCSIKA	SNGVFGDPYK
Consensus		.	.	: . C	G :. .
	110	120	130	140	150
Ga_Lectin	---VQGPQSF	VIDTNGYVTE	QWLGATDQSS	EGTWNWLWIH	PS-----
Aj_Lectin	NVEYQFLREL	NKASDPQDSM	FWIGLTDIRK	EGTWVWSDGS	AVD-----
Pm_Lectin	---VYALKSF	VIDTAAEVTE	AWLGATDQSS	EGTWNWLDGR	PV-----
Pp_Lectin	GKKKRLLIKY	SCAHNGETST	KIANGKEHTS	SASLKCSGIK	YTIRVIEAEY
Consensus	.	:	. . :	. . .	
	160	170	180	190	200
Ga_Lectin	--HSDWAGGQ	PDK---AGGN	EDCLDLRTKI	RVDGNDYQCG	VASIFVCQYN
Aj_Lectin	--FTTWNPQG	PDD---WQGN	EDCVHANVPE	QKNWNDVDCS	TPYRFICALR
Pm_Lectin	--ASDWAGGQ	PDD---AGGN	EDCLDLRTKW	HPTLNDYQCG	VAQHFVCQYN
Pp_Lectin	GISQRWWDGT	SKVRKMC DGL	KECMVPAVN-	-YMFGDPAVG	KKKDLRVRYK
Consensus	W G ..	G ::C:	.	.D .	: .
	206	Identity (%)			
Ga_Lectin	ARX-	100			
Aj_Lectin	SNAAGKX	22			
Pm_Lectin	AX-----	68			
Pp_Lectin	CTSX---	24			
Consensus	.				

Figure 5. Comparison of the aligned amino acid sequences of lectin of of red algae *Gelidium amansii* (Ga_Lectin) with Crustacea *Penaeus monodon* (Pm_Lectin, Genebank DQ078266), Chordata *Anguilla japonica* (Aj_lectin, Genebank: AB060538), and mollusca *Pteria penguin* (Pp_lectin, Genebank: AB037167). The complete amino acid sequences of four proteins of marine organisms are shown. The positions of the amino acid sequence are indicated on the ends. Amino acid residues that are identical in two is marked with : and three is marked with . and analogous amino acids in all these proteins are indicated by bold letter.

Expression and purification of the recombinant lectin protein in E. coli

The coding sequence of cDNA lectin was expressed as part of the pGEX-2T fusion protein, GST-lectin. The one-step purification procedure as described in (Ahmad *et al.* 1999) was

used to improve solubility of the GST-lectin fusion protein. Based on SDS-PAGE (14%) in Figure 6, the molecular mass of the GST-lectin fusion protein product was ≈ 47 kDa (fusion of the molecular mass of the lectin alone was ≈ 21 kDa with the GST was ≈ 26 kDa) was dramatically accumulated in *E.coli* BL 21 cell containing the pGEX-2TK lectin plasmid with induction by 60 μ M IPTG and could be purified to more than 95% homogeneity, using glutathione agarose beads (see lane 3 in Figure 6).

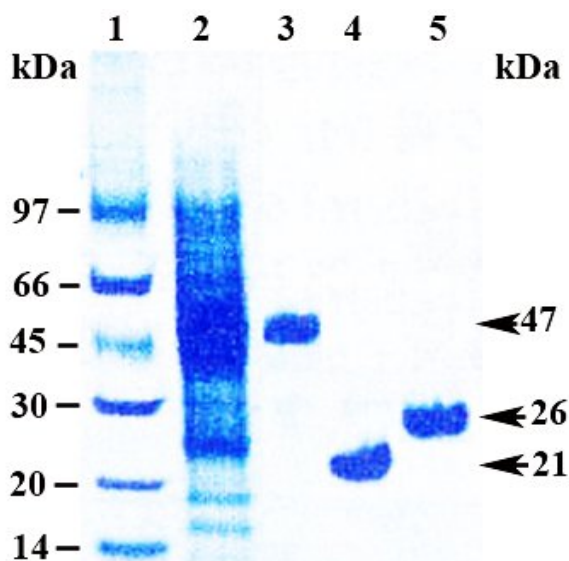


Figure 6. Electrophoresis SDS-PAGE (14%) analysis of the recombinant GST-lectin fusion protein and native lectin protein. Lane 1, Protein marker; Lane 2, Whole extract protein in *E. coli* cell containing pGEX-2TK lectin cDNA; Lane 3, Recombinant GST-lectin fusion protein; Lane 4, native lectin protein and Lane 5, GST protein alone.

Anti-mycobacterial activity of the recombinant GST-lectin fusion protein

The anti-mycobacterial activity of the recombinant GST-lectin fusion protein against *M. tuberculosis* test was performed with LJ medium and Middlebrook 7H10-OADC agar plate assay. The combinations of recombinant lectin and streptomycin were tested in the anti-mycobacterial assay described above by a checkerboard method in order to identify those combinations that could enhance bioactivity effect. The recombinant lectin alone was not able to exhibit bacterial growth, although native lectin protein contained anti-mycobacterial activity (Figure 7), and was in accordance with the results of the whole extract and protein fraction in 40-60% saturation that showed significant inhibition of growth of *M. tuberculosis* (Figure 1). This difference in results might indicate that whole extract and 40-60% fraction protein could contain other compound(s) that increase synergical effect on growth of *M. tuberculosis* and/or the recombinant lectin had structural differences in properties compared to native lectin or the incorrect folding of recombinant lectin protein. The combinations with the streptomycin was able to exhibit enhanced mycobacterial effect not only from native lectin but also recombinant lectin

at the lowest concentration (5 $\mu\text{g/mL}$) of anti-mycobacterial agent (Figure 8). In conclusion, our results demonstrated that recombinant lectin was active against *M. tuberculosis* when used in

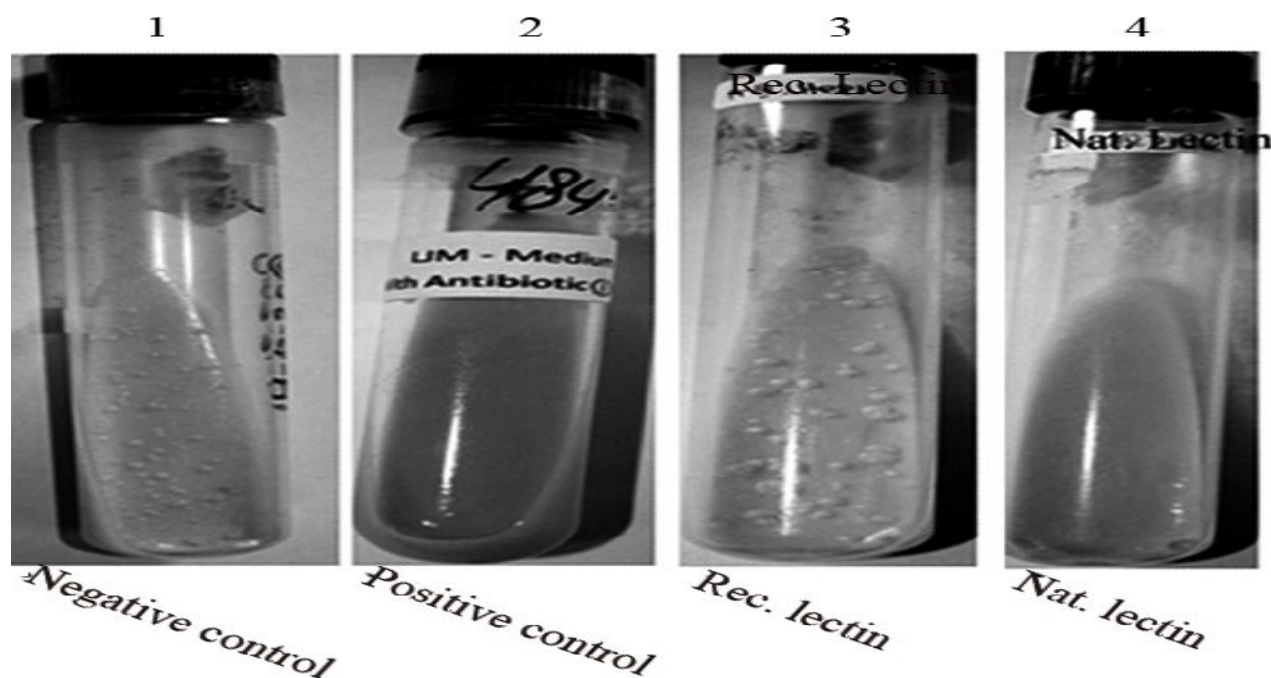


Figure 7. Effect of recombinant and native lectin protein alone on growth inhibition of *M. tuberculosis* H37Rv (ATCC 27294) strain in Löwenstein-Jensen medium bottle.

combination with other antimicrobial agents (streptomycin), and increased the sensitivity of the anti-TB drug. This result suggested a future use of the protein in association with clinically used drugs in TB therapy.

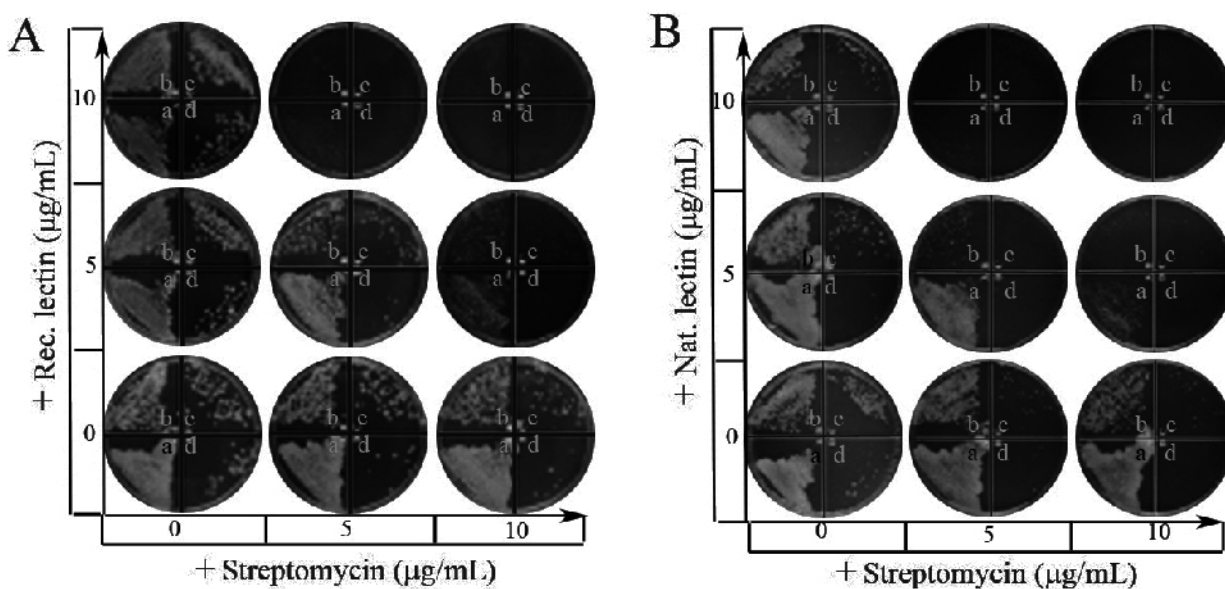


Figure 8. Effect of recombinant lectin protein (A) and native lectin protein (B) in combination with streptomycin in Middlebrook 7H10-OADC agar plate on growth inhibition of *M. tuberculosis* H37Rv (ATCC 27294) strain. Region a, b, c, and d correspond to serial dilution (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) of an *M. tuberculosis* H37Rv settling culture ($\text{OD}_{600}=0.2$).

DISCUSSION

In the current study, we purified and cloned a novel anti-mycobacterial lectin protein from a red algae *Gelidium amansii* of the class Rhodophyceae. Recently, Liao *et al.* (2003) found the strong antibiotic lectin from marine algae against vibrios. Further, Schröder *et al.* (2003) identified and cloned anti-bacterial lectin protein from sponge *S. domuncula* with activity against the gram-negative *E. coli* and the gram-positive *S. aureus*. Very recently British, Irish and Turkish seaweeds of several green, brown, and red algae were reported with anti-mycobacterial effects (Allmendinger *et al.* 2010; Spavieri *et al.* 2010 Selçuk *et al.* 2011). A few of their genes encoding some proteins that function similarly to antibodies have been cloned, which is critical for further studies; however, no lectin gene or protein sequence was reported from tropical algae to date, especially from Indonesian territory. In this work, a novel anti-mycobacterial lectin from red algae *Gelidium amansii* was characterized, its cDNA was cloned and expressed in *E. coli* as recombinant protein. Our results indicated that lectin from red algae *Gelidium amansii* acts primarily on acid-fast bacteria especially against *M. tuberculosis*. This results was in accordance with previous study that hot water extracts from *Gelidium capillaries* and *Gelidium amansii* showed some inhibition towards *M. avium* and *M. tuberculosis*, respectively (Kamimoto, 1956).

Molecular structures and the aligned amino acid sequences of different lectins isolated from various crustaceans, chordate, mollusca, and marine algae are quite different. Most purified algae lectins are multimeric proteins, containing a unique subunit ranging from 20 to 40 kDa (Oliveira *et al.*, 2002). We found red algae *Gelidium amansii* lectin to be a monomer with molecular weight of 21 kDa; however, its ability to function as an anti-mycobacterial agent suggested that it may be oligomeric and correct structure although it might not be covalent bond, disulphide-bonded forms were seen on gels. In addition, we concluded that red algae *Gelidium amansii* lectin was not a glycoprotein on the basis of the results of various experiments and sequence analysis. Analysis of a recombinant product of lectin protein in *E. coli* showed no activity when tested alone; required glycosylation or the other post translational modification. Most lectins are glycoproteins, but the glycosyl in lectins may not be involved in carbohydrate recognition.

Individual animal species probably contain several lectins, including C-type lectins of different specificities, for detecting a variety of pathogens. Recognition of microorganisms by

these lectins may trigger different immune responses. Although multiple lectins have been isolated from some marine invertebrates, such as tunicates (Nair *et al.* 2000), sponges (Miarons *et al.* 2000), crustaceans (Cominetti *et al.* 2002), echinoderms (Hatakeyama *et al.* 1994), and fish egg (Jimbo *et al.* 2007), no further study on its gene was published until now in marine algae, especially from Indonesian territory. Moreover, we have purified, cloned and identified an anti-mycobacterial lectin gene from red algae *Gelidium amansii* in Barang Lompo Island, South Sulawesi Province, Indonesian territory and found that lectin protein contained direct or indirect anti-mycobacterial activity. The biological function of lectin protein in red algae *Gelidium amansii* itself is undiscovered, but this lectin plays some roles in the self-defence system of red algae *Gelidium amansii*. It should be noted that lectin protein from marine invertebrates interacted with not only Gram-negative but also Gram-positive bacteria (Schröder *et al.* 2003), including acid-fast bacteria. The bioactivity of lectin may appear to function as a pattern recognition protein specific for Gram-negative and acid-fast bacteria through its interaction with Lipopolysaccharide (LPS), and also as an opsonin to increase the efficiency of hemocyte phagocytosis. Lectin protein combined with LPS causes rapid agglutination of Gram-negative and acid-fast bacteria, and lectin protein combined with phagocytes causes sensitization of the phagocytes to bacteria.

We used *in vitro* systems in this study; further studies need to be performed regarding anti-mycobacterial activity and repeated in an *in vivo* system. Therefore, it is necessary to carry out some more studies in animal models of tuberculosis to confirm these findings. These works might provide a significant foundation for further research on the anti-mycobacterial action in molecular and cellular level of lectin protein from marine algae and also it would be helpful to recognize lectin's role in the marine invertebrate innate immunity.

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